New Phytologist

Research

Gaining or cutting SLAC: the evolution of plant guard cell signalling pathways

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Received: *10 June 2024*
Accepted: *8 September 2024*

New Phytologist (2024)
doi: 10.1111/nph.20172

Key words: abscisic acid, angiosperms, ferns, OPEN STOMATA 1, plant evolution, seed plants, stomata, S-type anion channel (SLAC/SLAH) family.

Summary

- The evolution of adjustable stomatal pores, enabling CO_2 acquisition, was one of the most significant events in the development of life on land. Here, we investigate how the guard cell signalling pathways that regulate stomatal movements evolved.
- We compare fern and angiosperm guard cell transcriptomes and physiological responses, and examine the functionality of ion channels from diverse plant species.
- We find that, despite conserved expression in guard cells, fern anion channels from the SLAC/SLAH family are not activated by the same abscisic acid (ABA) pathways that provoke stomatal closure in angiosperms. Accordingly, we find an insensitivity of fern stomata to ABA. Moreover, our analysis points to a complex evolutionary history, featuring multiple gains and/or losses of SLAC activation mechanisms, as these channels were recruited to a role in stomatal closure.
- Our results show that the guard cells of flowering and nonflowering plants share similar core features, with lineage-specific and ecological niche-related adaptations, likely underlying differences in behaviour.

Introduction

Land plants evolved from a green algal ancestor, which conquered dry land over 500 million years ago (Morris *et al.*, 2018). The adjustable stomatal pore, which enables CO_2 acquisition in cuticle-covered tissues, is a key innovation that likely helped plants to thrive on land. The stomatal pore is usually flanked by two guard cells that regulate stomatal aperture, found in all major land plant lineages except liverworts (see Duckett & Pressel, 2018). In vascular plants (lycophytes, ferns, gymnosperms and angiosperms), mature stomata are able to close and reopen in response to internal and environmental signals and have an important role in preventing excessive water loss. In contrast to those of vascular plants, stomata in mosses and hornworts appear important for drying spores and sporophyte dehiscence (Duckett *et al.*, 2009; Chater *et al.*, 2016; Renzaglia *et al.*, 2017). Moss stomata may respond to some stimuli during early development, including exogenous ABA application (Chater *et al.*, 2011), but develop mechanical restrictions that prevent closure at maturity (Merced & Renzaglia, 2014; Duckett & Pressel, 2022). Mosses in the genus *Sphagnum* have pseudostomata, which lack a stomatal pore but possess two guard cells that collapse irreversibly, promoting spore desiccation (Duckett *et al.*, 2009; Merced, 2015). Hornwort stomata open once only through irreversible guard cell collapse (Renzaglia *et al.*, 2017).

Stomata have been most thoroughly studied in angiosperm species, which have revealed the specialised multisensory signalling pathways, cell wall and membrane components that enable guard cells to undergo rapid directed volume changes for active stomatal movements. Stomatal closure in response to dehydration stress is mediated by the hormone abscisic acid (ABA), synthesised in leaves and even guard cells themselves during drought stress (e.g. Mittelheuser & Van Steveninck, 1969; Bauer *et al.*, 2013; McAdam *et al.*, 2016b). In diverse angiosperms, orthologs of the S-type anion efflux channel SLOW ANION CHANNEL 1 (SLAC1) are activated upon phosphorylation by the serine/threonine protein kinase OPEN STOMATA 1 (OST1) from the SNF1-related protein kinase 2 (SnRK2) family,

in an ABA-dependent, calcium-independent manner (Vahisalu *et al.*, 2008; Geiger *et al.*, 2009; Müller *et al.*, 2017; Y. Li *et al.*, 2022). In addition, SLAC1 and a member of the same ion channel family – SLAC1 HOMOLOGUE 3 (SLAH3) – are sensitive to calcium signals via activation by calcium-dependent protein kinases (CPKs) and/or calcineurin B-like protein (CBL)-interacting protein kinases (CIPKs) (Geiger *et al.*, 2010, 2011; Scherzer *et al.*, 2012; Maierhofer *et al.*, 2014a; Huang *et al.*, 2023). ABA can also induce calcium signals in guard cells before stomatal closure (McAinsh *et al.*, 1990), and the CPKs and CIPKs form an OST1-independent branch of the ABA-signalling pathway (Mori *et al.*, 2006; Geiger *et al.*, 2010; Scherzer *et al.*, 2012; Brandt *et al.*, 2015). Other members of the SLAC/SLAH family – SLAH1, SLAH2 and SLAH4 – have apparent roles in translocation of Cl^- and NO_3^- ions in roots and vascular tissues for nutrition and salinity tolerance (Maierhofer *et al.*, 2014b; Cubero-Font *et al.*, 2016; Qiu *et al.*, 2016).

Abscisic acid perception and signalling components appear largely conserved between land plants (e.g. Bowman *et al.*, 2017; Xiao *et al.*, 2018; Shinozawa *et al.*, 2019; Sun *et al.*, 2019; Nibau *et al.*, 2022). However, it is currently debated whether or not ABA also has a conserved function in stomatal closure in seedless plant groups (see Sussmilch *et al.*, 2019b; McAdam *et al.*, 2021; Clark *et al.*, 2022; Chater, 2024). There are numerous reports of stomatal responses to applied ABA in seedless lineages including mosses, lycophytes and ferns, dependent on conditions (Chater *et al.*, 2011; Ruszala *et al.*, 2011; Hõrak *et al.*, 2017). Coupled with phylogenomic studies indicating gene losses in bryophytes, these findings have led to the popularity of the theory that land plant stomata had a complex, ABA-sensitive ancestral state (e.g. Clark *et al.*, 2022). In support of a more gradual evolution of ABA-mediated stomatal control mechanisms, so far only seed plants have been shown capable of closing stomata in response to elevated endogenous ABA levels (Brodribb & McAdam, 2011; McAdam & Brodribb, 2012), coinciding with the evolutionary expansion of the gene families involved in ABA-dependent stomatal closure (Bowles *et al.*, 2022). Intriguingly, experiments in *Xenopus* oocytes have revealed that SLAC/SLAH channels from the alga *Klebsormidium nitens*, the liverwort *Marchantia polymorpha*, the lycophyte *Selaginella moellendorffii* (all SmSLAC homologues tested) and the fern *Ceratopteris richardii* (two CrSLAC homologues tested) are not activated by OST1 kinases from the respective species (McAdam *et al.*, 2016a), in contrast to angiosperm SLAC1 channels (e.g. Geiger *et al.*, 2009; Müller *et al.*, 2017; Qi *et al.*, 2018; Schäfer *et al.*, 2018). However, a functional OST1-SLAC pair was isolated from the moss *Physcomitrium/Physcomitrella patens*, indicating that at least one bryophyte also has a SLAC homologue that can be activated by an ABA-sensitive kinase (Lind *et al.*, 2015).

In this study, we investigated the evolution of guard cell signalling pathways. We performed RNA-seq experiments to find out what makes guard cells special compared with other leaf cells in vascular plants, and what transcriptional features separate angiosperm and fern guard cells. For this, we used two fern species, *C. richardii* and *Polypodium vulgare*, in comparison with the angiosperms *Arabidopsis thaliana* and barley (*Hordeum vulgare*). *C. richardii* is an aquatic fern with a long history as a model for genetics (e.g. Hickok *et al.*, 1987, 1995), and *P. vulgare* is an historical stomatal research model capable of rapid, reversible stomatal responses to air humidity (Lange *et al.*, 1971; Stevens & Martin, 1977; Lösch, 1979). Our results indicate that these fern guard cells express homologues of many important angiosperm guard cell genes, including those encoding SLAC/SLAH ion channels and kinases from the SnRK2, CPK and CIPK families. We tested the activity of the guard cell-expressed fern SLAC proteins and examined the evolution of SLAC activity in land plants using additional bryophyte and seed plant models. We found that fern SLAC proteins are not activated by the same ABA-dependent pathways as angiosperms. Our results reveal the diversity of guard cell regulatory mechanisms in plants alive today, while shedding light on how these have evolved over the past 500 million years of plant life on land.

Materials and Methods

ABA electro-infusion

Polypodium vulgare L. plants were obtained from the Würzburg Botanical Gardens and grown in a glasshouse with *Nicotiana tabacum* L. cv SR1, under natural light extended with HQL-pressure lamps (Powerstar HQI-E, 400 W; Philips, Eindhoven, the Netherlands) at a 12 h : 12 h, day : night cycle. Extracellular application experiments (current ejection) with microelectrodes were conducted with intact plants. The adaxial side of leaves was attached with double-sided adhesive tape to a Plexiglas holder in the focal plane of an upright microscope (Axioskop 2FS; Zeiss). Stomata were visualised with a water immersion objective (W Plan-Apochromat, 40×/0.8, or 63×/1.0; Zeiss) dipped into bath solution (5 mM KCl, 0.1 mM $CaCl_2$ and 5 mM K-citrate, pH 5.0) on the abaxial surface of the leaf. Microelectrodes were put in contact with the cell wall of stomata with a piezo-driven micro-manipulator (MM3A; Kleindiek Nanotechnik, Reutlingen, Germany), as described previously (Huang *et al.*, 2019). The microelectrodes were prepared from borosilicate glass capillaries (inner diameter, 0.58 mm; outer diameter, 1.0 mm; Hilgenberg) and filled with solution containing 1 mM Lucifer Yellow (LY) and 1 mM ABA, or only 1 mM LY (control). The ABA concentration in the electrodes was 20 times higher than that used by Huang *et al.* (2019) and a −1 nA ejection current was applied, instead of −0.8 nA used by Huang *et al.* (2019). In comparison with the analysis of Huang *et al.* (2019), current ejection will have resulted in a local ABA concentration of 25 µM, which decreases by 50% over 1 µm.

The stomatal movements and ejection of LY were monitored with a charge-multiplying CCD camera (QuantEM; Photometrics, Tucson, AZ, USA). The fluorescent probe was excited with a Hg metal-halide lamp (HXP120; Leistungselektronic JENA, Jena, Germany), through a band pass filter of 430/24 nm (ET 430/24; Chroma Technology Corp., Bellows Falls, VT, USA). A dichroic mirror (495 nm LP) guided the excitation light through the objective, while the fluorescent light was filtered with

an emission band pass filter (520/30 nm; BrightLine, Semrock, West Henrietta, NY, USA). The filters could be rapidly moved in and out of the light path, with filter wheels of a spinning disc confocal unit (CARV II; Crest Optics, Rome, Italy) that was mounted to the camera port of the upright microscope. Image acquisition was conducted with VisiView software (Visitron, Puchheim, Germany) and analysed the ImageJ/Fiji software package (Schindelin *et al.*, 2012).

Arabidopsis RNA-seq

Arabidopsis thaliana (L.) Heynh. plants were grown in semi-sterilised soil (treated for 20 min at 100°C), cultivated in climate chambers (Binder KBWF 720) in a 12-h day–night rhythm (22/16°C, 60% RH) and were illuminated with 125 µmol m^{-2} s^{-1} white light. RNA was isolated from whole leaf samples and guard cell-enriched samples isolated by mechanical disruption as described previously (Bauer *et al.*, 2013), from 6 to 7 wk old plants. Library preparation and RNA-seq were carried out as described in the Illumina TruSeq Stranded mRNA Sample Preparation Guide, the Illumina NextSeq 500 System Guide (Illumina Inc., San Diego, CA, USA), and the KAPA Library Quantification Kit – Illumina/ABI Prism User Guide (Kapa Biosystems Inc., Woburn, MA, USA).

Briefly, 250 ng of total RNA was used for purifying the poly-A-containing mRNA molecules using poly-T oligo-attached magnetic beads. Following purification, the mRNA was fragmented to an average insert size of 200–400 bases using divalent cations under elevated temperature (94°C for 4 min). Next, the cleaved RNA fragments were reverse transcribed into first-strand cDNA using reverse transcriptase and random hexamer primers. Actinomycin D was added to improve strand specificity by preventing spurious DNA-dependent synthesis. Blunt-ended second strand cDNA was synthesised using DNA Polymerase I, RNase H and dUTP nucleotides. The incorporation of dUTP, in place of dTTP, quenched the second strand during the later PCR amplification. The resulting cDNA fragments were adenylated at the 3′ ends, the indexing adapters were ligated and subsequently specific cDNA libraries were created by PCR enrichment. The libraries were quantified using the KAPA SYBR FAST ABI Prism Library Quantification Kit. Equimolar amounts of each library were sequenced on a NextSeq 500 instrument using two 150 Cycles High Output and one 150 Cycles Mid Output Kits with the single index, paired-end (PE) run parameters. Image analysis and base calling resulted in .bcl files, which were converted into .fastq files with the bcl2fastq v.2.18 software. Library preparation and RNA-seq were performed at the service facility 'KFB – Center of Excellence for Fluorescent Bioanalytics' (Regensburg, Germany).

Barley RNA-seq

Barley (*Hordeum vulgare* L. cv. Barke) seeds were provided by a commercial supplier (Saatzucht J. Breun GmbH & Co. KG, Herzogenaurach, Germany) and cultivated at 22/16°C and 50 ± 5% RH at a 12 h : 12 h, day : night cycle and a photon flux density of 500 mmol m^{-2} s^{-1} white light (400 W; Philips Master T Green Powers). For guard cell-enriched samples, epidermal peels were first isolated from the abaxial side of 8- to 12-d-old leaves, and then, subsidiary cells were disrupted with successive blender cycles in ice-cold water as described previously (Schäfer *et al.*, 2018). RNA was extracted using the NucleoSpin® RNA Plant Kit (Macherey-Nagel, Drueren, Germany). RNA isolation from whole leaves was performed similarly.

The extracted RNA was treated with RNase-free DNase (New England Biolabs, Ipswich, MA, USA). Quality control measurements were performed on a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and the concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Libraries were prepared with the TruSeq RNA Sample Prep Kit v2 (Illumina Inc.) using 1 mg of RNA and sequenced on a HiSeq 3000 (Illumina Inc.) resulting in a sequence depth of 35 million PE reads (2× 150 bp).

Fern RNA-seq

Ceratopteris richardii Brongn. wild-type (WT) strain Hn-n (Hickok *et al.*, 1995) was grown in controlled-condition glasshouse facilities under a 16-h photoperiod with supplemented natural light. *P. vulgare* plants (Common Polypody – winter hardy; Westdijk's, Kwekerijen) were grown in a growth chamber under a 12 h photoperiod, with 22°C : 16°C, day : night temperatures.

Four biological replicates, each comprising up to 100 mg tissues, were collected from both species for whole leaf samples comprising fully expanded sporophyte fronds lacking sporangia and guard cell-enriched samples. Guard cell-enriched samples were obtained from fully expanded fronds lacking sporangia, after removal of the main veins, by mechanical disruption of other cell types using successive 1–2 min blending cycles in deionised ice water with epidermal fragments collected by filtration through a 210 µm nylon mesh, according to a previously published method (Bauer *et al.*, 2013) optimised for *C. richardii* (3 cycles) and *P. vulgare* (5 cycles). Fluorescein diacetate staining (Widholm, 1972) was used to confirm guard cell viability and purity. In *P. vulgare*, 'leaf without epidermis' samples lacking guard cells were also obtained by using fine forceps to fully remove the abaxial epidermis from leaves.

Total RNA was extracted, treated with RNase-free DNase and quality control measurements were performed as described above. Library preparation and mRNA-seq after polyA-enrichment was performed by the Core Unit Systems Medicine (University of Würzburg). Both species were sequenced on the Illumina NextSeq500 platform using a single high-output flow cell for the 8 *C. richardii* samples and duplicate high-output flow cells for the 12 *P. vulgare* samples for 150 nt PE reads (300 cycles).

Transcriptome assembly and annotation

Transcriptomes for *P. vulgare* and *C. richardii* were assembled using Trinity (Grabherr *et al.*, 2011) with the trimmomatic option (Table S1). Coding regions were predicted with TransDecoder (https://github.com/TransDecoder/TransDecoder). Domains and GeneOntology annotations were predicted using InterPro (Blum

et al., 2021). To generate a level of annotation comparable to the fern data, all proteomes (Table S2) were annotated *de novo* using InterPro.

Differential expression

For the ferns, reads were mapped onto the reference transcriptomes by Salmon (Patro *et al.*, 2017). For *A. thaliana* and *H. vulgare*, the reads were mapped onto the reference genomes (Table S2) by RSEM (Li & Dewey, 2011). Differentially expressed genes were identified with DESeq2 (Love *et al.*, 2014).

Evolutionary reconstruction

Orthology relationships over all species (Table S2) were predicted using Orthofinder (Emms & Kelly, 2015). The last common ancestor for each orthogroup was calculated using the Bio::TreeIO Perl package. Expression groupings were classified into hierarchical categories of genes 'up'-regulated (1) expression higher in guard cells than leaves AND higher in whole leaves than leaves without guard cells – *P. vulgare* only, *P*adj ≤ 0.01; (2) expression higher in guard cells than leaves, *P*adj ≤ 0.01; (3) expressed in guard cell samples, baseMean ≥ 10; (4) expression negligible in guard cell samples, baseMean < 10 or 'down'-regulated (1) expression lower in guard cells than leaves AND lower in whole leaves than leaves without guard cells – *P. vulgare* only, *P*adj ≤ 0.01; (2) expression lower in guard cells than leaves, *P*adj ≤ 0.01 in guard cells relative to whole leaf samples based on the presence of one or more genes in the orthogroup meeting the category criteria (Table S3).

Phylogenetic analyses for streptophyte SLAC/SLAH and SnRK2 families

Genes were identified in the literature or by performing BLASTp searches using Arabidopsis protein sequences against the relevant genome or transcriptome assembly as indicated in Table S4, and confirmed with reciprocal BLASTp searches back against Arabidopsis and preliminary phylogenetic analyses. The maximum likelihood phylogenetic tree for the SLAC/SLAH family was calculated from a MAFFT alignment of full-length predicted protein sequences using PhyML v.3.0 at http://www.trex.uqam.ca/ with the JTT substitution model and 1000 bootstrap replicates (Guindon *et al.*, 2010). The maximum likelihood phylogenetic tree for the SnRK2 family was calculated using PhyML v.3.0 at http://www.atgc-montpellier.fr/phyml/ with SmartModel Selection (Guindon *et al.*, 2010; Lefort *et al.*, 2017), and 1000 bootstrap replicates, from a MAFFT alignment of predicted protein sequences trimmed using Gblocks via the online server at http://molevol.cmima.csic.es/castresana/Gblocks_server.html (Talavera & Castresana, 2007), with all options for reduced stringency selected. Full sequence and species details are given in Table S4.

Cloning and complementary RNA generation

Full-length coding sequence from *OST1*, *SLAC* and/or *CPK* homologues of *C. richardii* (Hn-n, obtained from J. Banks; leaf), *P. vulgare* (Common Polypody – winter hardy; Westdijk's, Kwekerijen; leaf), *Picea abies* (L) H. Karst. (plants maintained at the University of Tasmania; needles), *Ginkgo biloba* L. (plants maintained at the Würzburg Botanical Gardens; leaves), *Amborella trichopoda* Baill. (plants maintained at the University of Tasmania; leaves), *Sphagnum fallax* (H.Klinggr.) H. Klinggr. ('MN', obtained from S. Rensing, from an original sample from Dave Weston, Oak Ridge National Laboratory; gametophyte) and *Anthoceros agrestis* Paton ('Bonn', obtained from P. Szoevenyi; gametophyte) were isolated from cDNA generated from RNA from the tissues indicated using primers outlined in Table S5 and cloned into pNB1uYN and pNB1uYC expression vectors by uracil excision-based cloning (Nour-Eldin *et al.*, 2006). The details for all *A. thaliana* constructs, and *C. richardii CrSLAC1a*, *CrSLAC1b*, *CrGAIA1* and *CrPGAI* constructs have been published previously (Geiger *et al.*, 2009, 2010, 2011; Scherzer *et al.*, 2012; Maierhofer *et al.*, 2014a; McAdam *et al.*, 2016a). Where indicated, site-directed mutations were introduced using a modified USER fusion method, as previously described (Dadacz-Narloch *et al.*, 2011), using the primers outlined in Table S5. For functional analysis, complementary RNA (cRNA) was prepared with the AmpliCap-Max™ T7 High Yield Message Maker Kit (Epicentre Biotechnologies). Oocyte preparation and cRNA injection were performed as described previously (Becker *et al.*, 1996). For oocyte bimolecular Fluorescence Complementation (BiFC) and electrophysiological experiments, 10 ng of each SLAC:YFP^{CT} (vector pNB1uYC) and 10 ng of each OST1:YFP^{NT} or 5 ng of CPK:YFP^{NT} or AtCIPK23ΔEF:YFP^{NT} + AtCBL1:YFP^{NT} (vector pNB1uYN) cRNA or cRNA of the same genes cloned into the pNB1u vector without YFP fragments, were injected into *Xenopus laevis* (Daudin) oocytes.

Bimolecular fluorescence complementation experiments

Expression of BiFC constructs in oocytes was performed as described previously (Geiger *et al.*, 2009). Images were taken with a confocal laser scanning microscope (Leica DM6000 CS; Leica Microsystems CMS GmbH, Wetzlar, Germany) equipped with a Leica HCX IRAPO L25×/0.95 W objective. Images were processed (low pass–filtered and sharpened) identically with the image acquisition software LAS AF (Leica Microsystems CMS GmbH).

Double-electrode voltage-clamp studies

Oocytes were perfused with MES/Tris-based buffers containing 10 mM MES/Tris (pH 5.6), 1 mM $Ca(gluconate)_2$, 1 mM $Mg(gluconate)_2$, 1 mM $LaCl_3$ and 100 mM NaCl, $NaNO_3$ or Na(gluconate). To balance ionic strength, changes in chloride or nitrate concentration were compensated with Na(gluconate). The standard voltage protocol was as follows: starting from a holding potential (V_H) of 0 mV, single 50 ms-voltage pulses were applied in 10 mV decrements from +70 to −150 mV, with instantaneous currents extracted right after the voltage jump from the holding potential.

Results

Fern and angiosperms share conserved guard cell expression of core genes but show lineage-specific expression patterns for others

For insight into the transcriptional features of fern guard cells compared with those of angiosperms, we performed RNA-seq experiments using Arabidopsis, barley, *C. richardii* and *P. vulgare*. For each species, we enriched guard cells by mechanical isolation of epidermal fragments through disruption of other cell types (Bauer *et al.*, 2013) and identified genes significantly ($P \leq 0.01$) up- or downregulated in guard cells relative to whole leaves. To reconstruct the evolutionary history of these genes, we extended our species set with representative models from major land plant lineages and streptophyte algae. Prediction of orthogroups based on this diverse dataset allowed us to identify genes derived from a single common ancestor. We classified three types of orthogroups of interest containing genes up- or downregulated in guard cell samples: (1) shared (genes differentially expressed in all four species), (2) angiosperm-specific (Arabidopsis and barley only) and (3) fern-specific (*C. richardii* and *P. vulgare* only). We found that the majority of these differentially expressed orthogroups included green algal genes, suggesting an ancient evolutionary origin and likely presence in an algal ancestor of land plants before terrestrialisation, predating specialisation in different embryophyte cell types including guard cells (Fig. 1a–c).

To gain insight into the function of the orthogroups differentially expressed in guard cells, we compared domain content and Gene Ontology (GO) classifications. We found that guard cells of all four vascular plant species shared expression of genes associated with ATP-binding, protein kinase activity/phosphorylation and membrane components (Figs 1d, S1; Table S3). Specific examples of shared guard cell orthogroups included *POLYGALACTURONASE INVOLVED IN EXPANSION* (*PGX*) family genes associated with pectin degradation and guard cell wall mechanics (Rui *et al.*, 2017; Yi *et al.*, 2018), and aquaporins from the plasma intrinsic proteins (PIP) family, which transport water and other solutes including CO_2 across the plasma membrane (Bienert *et al.*, 2018).

Angiosperm-specific guard cell orthogroups were frequently classified by GO terms associated with energy transfer, signalling and/or membrane components (Fig. 1d; Table S3). This is in line with further specialisation for these types of genes in angiosperms, where stomatal movements are tightly regulated by complex signalling pathways (see Sussmilch *et al.*, 2019b). Examples of angiosperm-specific guard cell orthogroups included cellulose synthase/cellulose synthase-like (CESA/CSL) superfamily genes, and C-type lectin receptor-like kinase genes linked to immune responses (Sun *et al.*, 2020).

Conversely in fern guard cells, genes associated with protein kinase activity and phosphorylation were commonly downregulated (Figs 1d, S1; Table S3), including genes from the Cytosolic ABA Receptor Kinase (CARK) family involved in ABA signalling in Arabidopsis (see L. Zhang *et al.*, 2018; X. Li *et al.*, 2022). Instead, orthogroups associated with oxidation–reduction and metabolic processes were over-represented in ferns, including orthologs of Arabidopsis mitochondrial glutamate dehydrogenase genes *GDH1/2/3* which feed the tricarboxylic acid (TCA) cycle to release stored energy (Fontaine *et al.*, 2012), and phosphoglucomutases *PGM1/2/3* involved in starch/sucrose/cell wall synthesis (Malinova *et al.*, 2014). This may reflect the higher abundance of chloroplasts in fern guard cells (Fig. 1c; Voss *et al.*, 2018), which work together with mitochondria to supply cells with energy and metabolites.

Fern guard cells express homologues of ABA biosynthesis and signalling genes

Genetic signalling pathways controlling stomatal movements have been well described in angiosperms, but the extent to which these are conserved in guard cells of other plant lineages is less well understood. Using our angiosperm and fern guard cell RNA-seq data, we looked specifically at homologues of key angiosperm genes to identify those that are expressed and/or upregulated in guard cell relative to whole leaf samples. For *P. vulgare* – which has stomata limited to the abaxial leaf surface – we also compared whole leaf samples to leaf samples with abaxial epidermis removed (lacking guard cells), to identify which genes were expressed at both (1) a higher level in guard cells than whole leaves and (2) a higher level in whole leaves than leaf samples lacking guard cells.

Consistent with angiosperms, homologues of the conserved guard cell specification gene *FAMA* (Ohashi-Ito & Bergmann, 2006; Chater *et al.*, 2016), showed significantly higher expression in fern guard cells than whole leaf samples (Fig. 2; Table S6). Similarly, both ferns showed expression of homologues of ABA biosynthesis and signalling genes in the guard cell samples, similar to the angiosperms (Figs 2, S2; Table S6). Although homologues of ABA2 – a short chain dehydrogenase (SDR) dedicated to the ABA biosynthesis pathway (Cheng *et al.*, 2002; González-Guzmán *et al.*, 2002) – are restricted to angiosperms (Moummou *et al.*, 2012; McAdam *et al.*, 2015; Sussmilch *et al.*, 2017), other SDRs are likely able to catalyse this step, and some closely related SDRs were expressed in the fern guard cell samples (Fig. S2; Table S6). Among the ABA-signalling pathway, some genes encoding phosphatases from protein phosphatase type 2C clade A (PP2CA) and kinases from the CPK and CBL-interacting kinase (CIPK) families were expressed at higher levels in guard cell than whole leaf samples in the ferns (Fig. 2). Other ABA-signalling genes including members of the *OST1* subclade of the *Sucrose Non-fermenting-1-Related Protein Kinase 2* (*SnRK2*) family were also nonspecifically expressed in the guard cells of the ferns, indicating their presence but likely nonspecific role in guard cells. Among the ion channels that are downstream of ABA-signalling genes in angiosperms, S-type anion channel genes from the SLAC/SLAH family were enriched in guard cells relative to whole leaves in all species. Overall, these results suggest that, similar to Arabidopsis and barley (Bauer *et al.*, 2013; Schäfer *et al.*, 2018), fern guard cells are equipped with the genetic toolkit required for ABA biosynthesis and signalling.

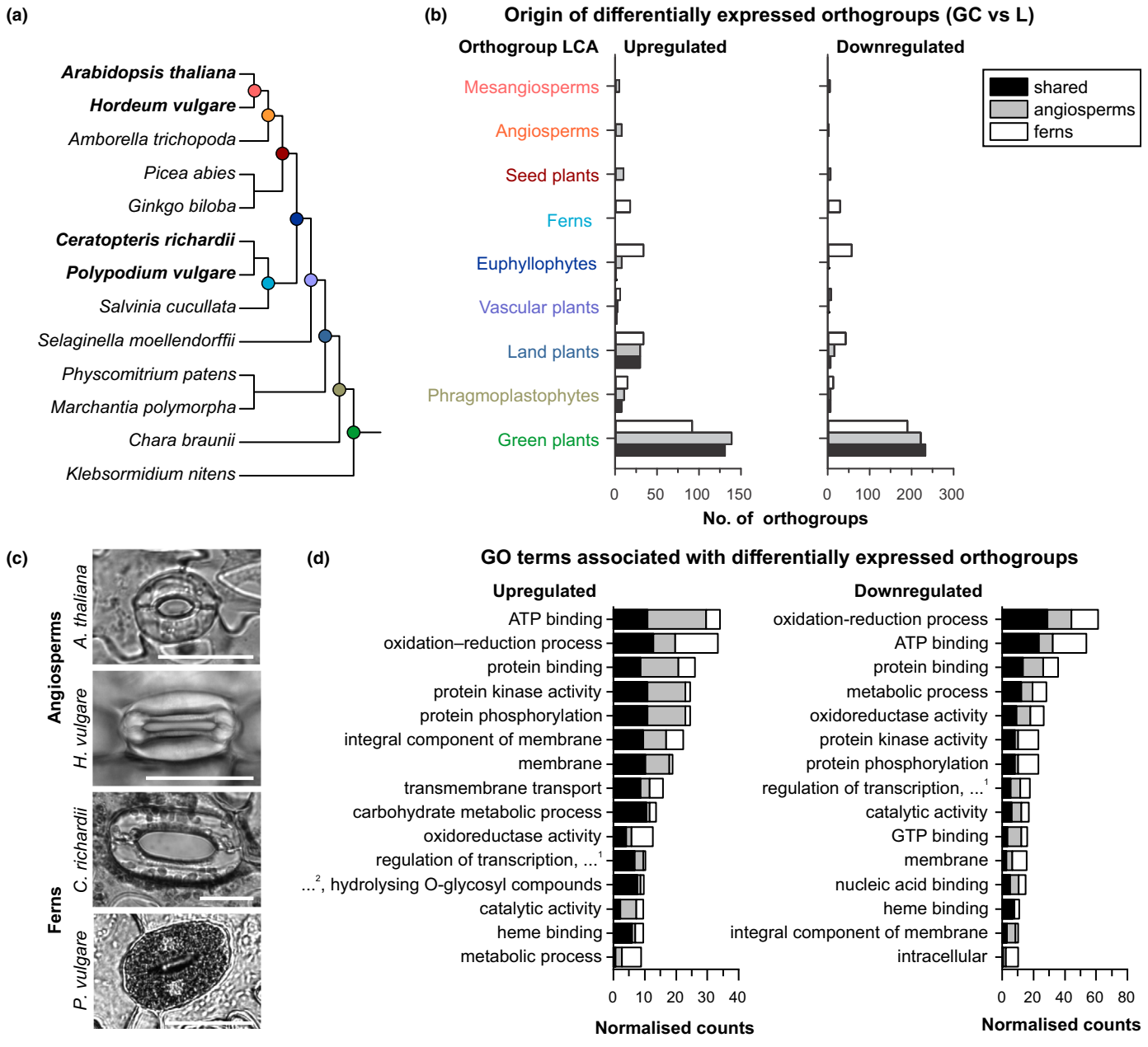

Fig. 1 There are core sets of genes shared between angiosperm (*Arabidopsis thaliana*, *Hordeum vulgare*) and fern (*Ceratopteris richardii*, *Polypodium vulgare*) guard cell transcriptomes with some lineage-specific differences. (a) Plant species included in RNA-seq experiments (bold) and orthology analyses, with phylogenetic relationships indicated (branch lengths not to scale). (b) Last common ancestor (LCA) nodes of orthogroups with significantly different expression between guard cell (GC) and whole leaf (L) samples in all angiosperm and fern species examined ('shared'; black), both angiosperm but neither fern species ('angiosperms'; grey), and both fern but neither angiosperm ('ferns'; white). (c) Photographs of guard cells of the species included in RNA-seq experiments (bar, 50 μm). (d) The top 15 most common Gene Ontology (GO) terms of orthogroups up- or downregulated in guard cell relative to whole leaf samples, with counts normalised to the total number of genes per orthogroup. Abbreviations are as follows: …[1] – 'DNA templated', …[2] – 'hydrolase activity'. See Supporting Information Fig. S1 for the most common annotated domains and Table S3 for all GO terms/annotated domains related to these orthogroups.

Fern SLAC homologues are not activated by ABA-signalling kinases

It is currently debated whether or not ABA has a role in stomatal closure in seedless plant groups including ferns (see Sussmilch *et al.*, 2019b; McAdam *et al.*, 2021; Clark *et al.*, 2022). To examine the stomatal responses of the fern *P. vulgare* to ABA, relative to those of the angiosperm tobacco (*N. tabacum*), we adapted a current-ejection method (Huang *et al.*, 2019) to apply ABA directly to guard cell walls in intact fern plants and monitor stomatal movements with an upright fluorescence microscope. Microelectrodes, filled with ABA and/or LY, were put in contact

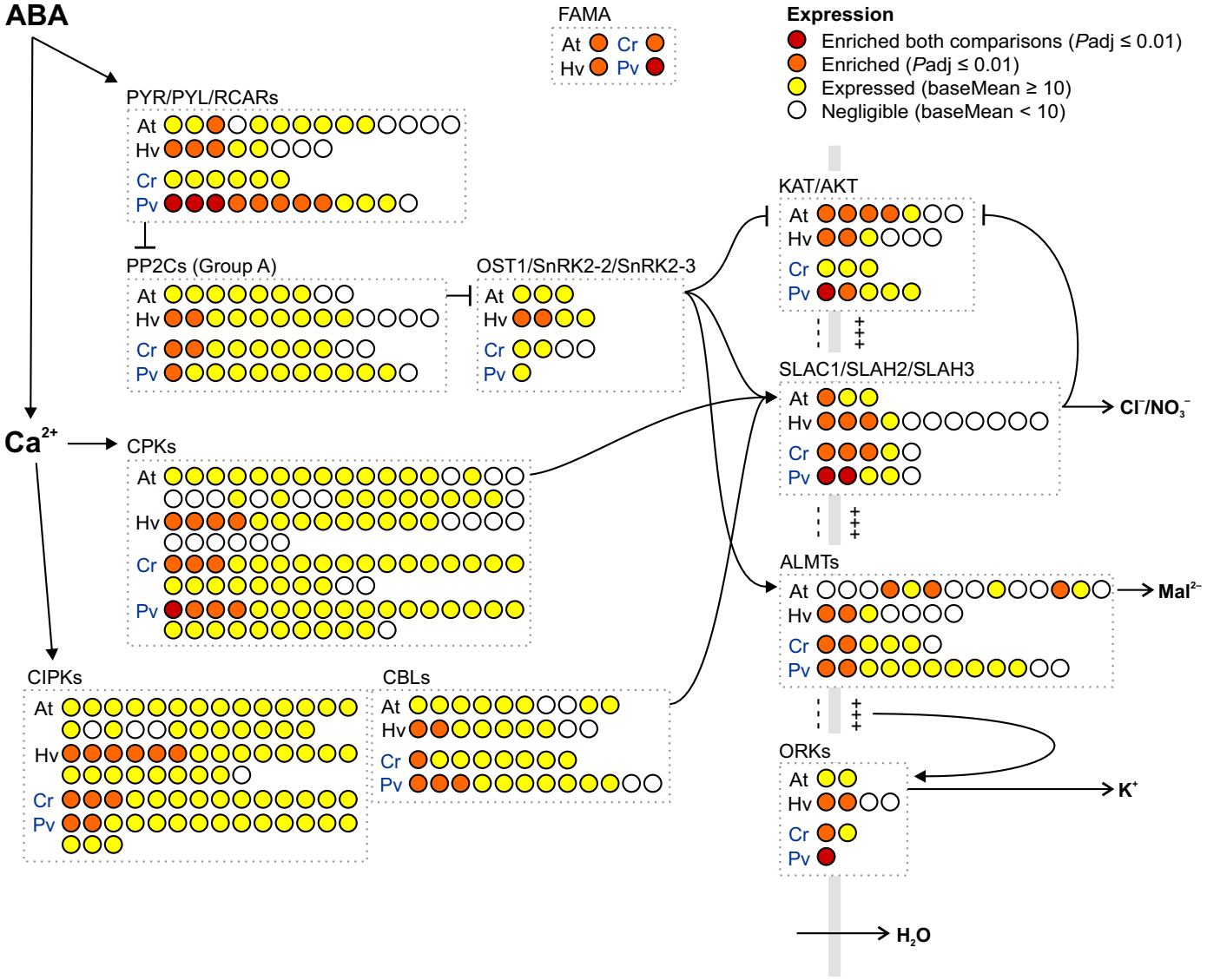

Fig. 2 Homologues of key abscisic acid (ABA)-signalling genes and ion channels are expressed in both angiosperm and fern guard cells. Groups of related genes are shown together in boxes with each circle representing a different gene, and colour coding representing relative expression in guard cell-enriched samples compared with whole leaves shown for angiosperm (At, *Arabidopsis thaliana*; Hv, *Hordeum vulgare*, barley) and fern (Cr, *Ceratopteris richardii*; Pv, *Polypodium vulgare*) models, as indicated. For *P. vulgare* samples only, whole leaf vs leaf samples without abaxial epidermis (thus guard cells removed) were also included and used to separate guard cell-enriched genes with a higher level of stringency (red; 'enriched both comparisons' = expression higher in guard cells than leaves and higher in whole leaves than leaves without guard cells). Homologues of the guard cell specification gene *FAMA* are also shown. The vertical grey line represents the guard cell plasma membrane with guard cell cytoplasm on the left and apoplast on the right. The role of gene products in guard cell signalling is indicated for key Arabidopsis genes (closed arrowhead, activation; blunt-ended line, deactivation; open arrowhead direction of ion/water flow); please note that this may not reflect the characteristics of all homologues. See Supporting Information Fig. S2 for ABA biosynthesis genes and Table S6 for details for all genes of interest examined.

with the apoplast, close to an open stoma. Application of a − 1 nA current for 1 min caused the appearance of fluorescence in guard cell walls of *P. vulgare* and tobacco (Fig. 3a), showing that negatively charged molecules can be applied to guard cells with this approach. The current ejection of LY into the guard cell walls did not provoke stomatal closure, while ABA provoked rapid stomatal closure in tobacco stomata (Fig. 3b,c). Based on the data in Huang *et al.* (2019), this procedure is estimated to result in a local concentration of 25 μM ABA at the tip of the electrode, which was in contact with the guard cell wall, with ABA concentration likely decreasing by 50% over *c.* 1 μm. To verify that stomata prechallenged with the control LY solution had not lost ABA responsiveness, we applied the stress hormone in a second ejection. ABA-induced stomatal closure was similar between guard cells pretreated with LY and those that were not (Fig. 3c). This confirmed that apoplastic application of ABA with microelectrodes causes rapid closure of tobacco stomata, just as previously reported for Arabidopsis (Huang *et al.*, 2019). In

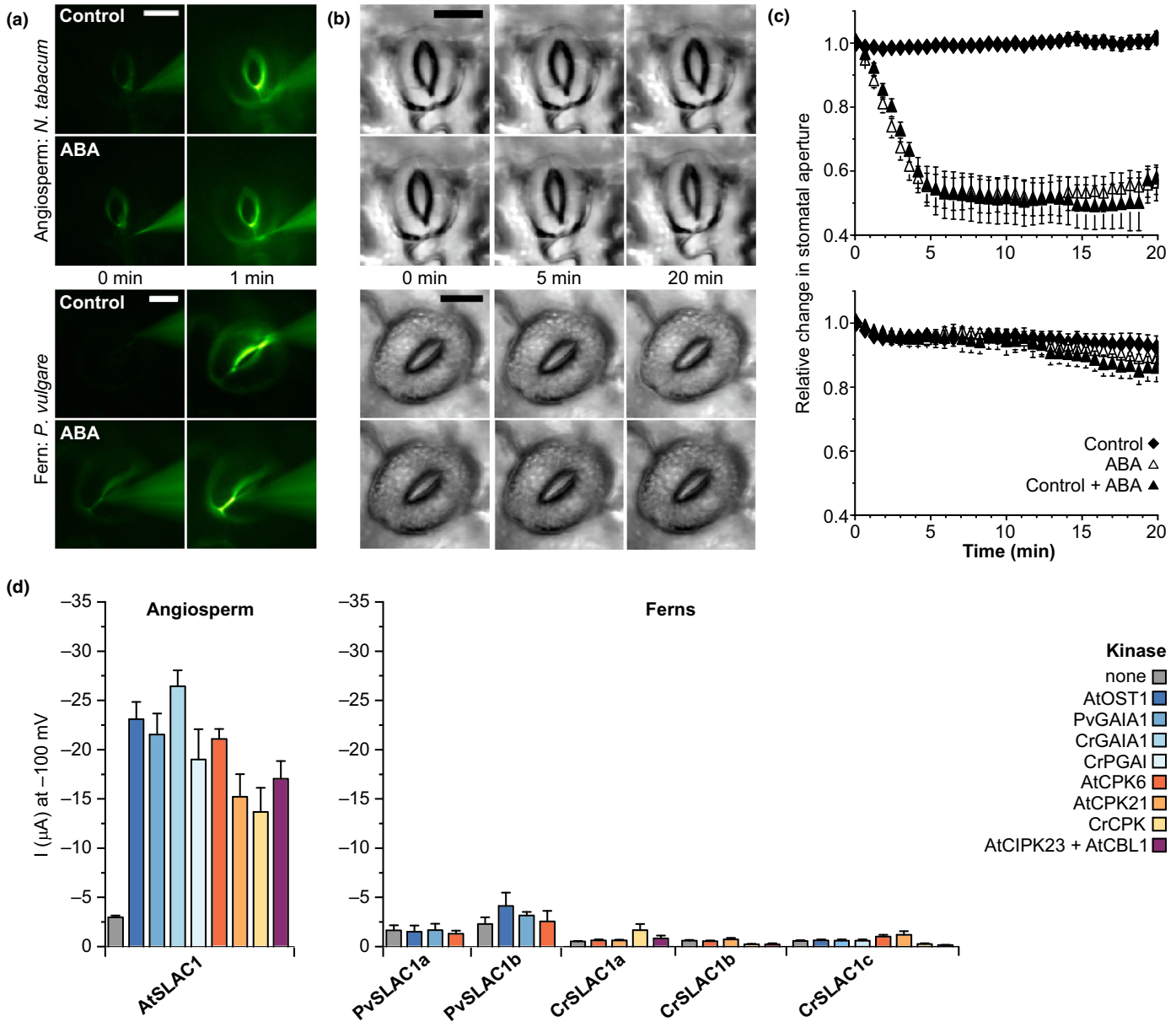

Fig. 3 Unlike angiosperm stomata, fern stomata are insensitive to abscisic acid (ABA), and fern guard cell SLOW ANION CHANNEL (SLAC) homologues are not activated by ABA-signalling kinases. (a) Fluorescent images of guard cells of the angiosperm *Nicotiana tabacum* (upper panels) and fern *Polypodium vulgare* (lower panels) at the start and 1 min after current ejection of 1 mM Lucifer Yellow (LY; control), or 1 mM LY and 1 mM ABA (bar, 10 μm). (b) Brightfield images of the guard cells shown in (a) up to 20 min after current ejection. (c) Normalised stomatal aperture, plotted against time for *N. tabacum* (upper graph) and *P. vulgare* (lower graph). Current ejection was performed at $t = 0$. Stomata treated with LY (control, diamonds) and subsequently to ABA (control + ABA, closed triangles), or to ABA only (ABA, open triangles; mean ± SEM, $n \geq 7$). (d) Mean whole-oocyte current measurements at −100 mV in nitrate-based solution with SLAC homologues from the angiosperm *Arabidopsis thaliana* and the ferns *P. vulgare* and *Ceratopteris richardii*, co-expressed with or without the indicated kinases in *Xenopus* oocytes (mean ± SEM, $n \geq 4$).

contrast with tobacco, no response to ABA could be observed for *P. vulgare* (Fig. 3b,c). These results indicate that, unlike angiosperms, stomatal response in the fern *P. vulgare* is not sensitive to ABA. These findings are consistent with those of others who have found a lack of stomatal responsiveness to ABA in ferns grown in glasshouse conditions (Brodribb & McAdam, 2011; McAdam & Brodribb, 2012; Cândido-Sobrinho *et al.*, 2022). Previous studies that have found a response of fern stomata to exogenous ABA applied to epidermal peels (Cai *et al.*, 2017) or by spraying onto leaves in plants under specific growth conditions (Hõrak *et al.*, 2017; Plackett *et al.*, 2021), have found the magnitude of response to be substantially smaller than that of angiosperms, indicative of a difference in ABA sensitivity between ferns and angiosperms.

We further investigated the molecular mechanism underlying this difference between fern and angiosperm stomatal ABA

responses. In angiosperms, orthologs of the anion channels SLAC1 and SLAH3 play a key role in stomatal closure responses to ABA, after activation by OST1-, CPK- or CIPK-type kinases (see Hedrich & Geiger, 2017). We previously found that two *C. richardii* SLAC/SLAH family members – CrSLAC1a and CrSLAC1b – were not activated by Arabidopsis kinase AtOST1 or the fern OST1-subclade kinases GAMETOPHYTES ABA INSENSITIVE ON A_{CE}1 (CrGAIA1) and PARALOG OF GAIA1 (CrPGAI) using the *Xenopus* oocyte expression system (McAdam *et al.*, 2016a). In this study, we identified four additional SLAC/SLAH family members (CrSLAC1c-f) expressed in *C. richardii* sporophytes (Fig. 2) and/or gametophytes (Atallah *et al.*, 2018). *CrSLAC1a-c* are expressed at higher levels in guard cells than whole leaf samples, as are two SLAC/SLAH family members in *P. vulgare* (*PvSLAC1a* and *PvSLAC1b*; Figs 2, S3, S4).

Using the *Xenopus* oocyte expression system together with the double-electrode voltage-clamp technique, we tested whether any of the newly isolated fern *C. richardii* and *P. vulgare* SLAC homologues are activated by kinases that activate AtSLAC1 from the angiosperm Arabidopsis. Specifically, we tested Arabidopsis ABA-signalling kinases, all guard cell-expressed kinases from the SnRK2 OST1-subclade in the two fern species, and a guard cell-enriched CPK from *C. richardii* (Figs 3d, S5). Each of these kinases interacted with the fern SLAC homologues (Fig. S6) and induced strong anion channel currents when co-expressed with AtSLAC1, but did not trigger increased currents with any of the *P. vulgare* guard cell-enriched SLAC homologues, or any of the *C. richardii* SLAC homologues expressed in currently available transcriptomes (Figs 3d, S3).

To confirm that the fern SLAC genes encode anion channels, we constructed CrSLAC1a F592A channel mutants, wherein the channel is gated open, similar to AtSLAC1 F450A (Chen *et al.*, 2010; J. Zhang *et al.*, 2018). We measured strong currents for the CrSLAC1a F592A mutant when co-expressed with AtOST1, but only very small background currents with CrSLAC1a F592A expressed alone (Fig. S3). This confirms that the fern anion channel can be activated in the absence of gating restrictions.

Overall, these results indicate that fern anion channels from the SLAC/SLAH family are not activated by the same kinases as angiosperm SLAC1 and SLAH3 orthologs that facilitate ABA-mediated stomatal closure. This finding is consistent with the lack of stomatal response to ABA we measured in *P. vulgare*. These results suggest that mechanisms for activation of these anion channels by ABA-dependent signalling kinases either (1) evolved after divergence of ferns from a common ancestor with seed plants, or (2) evolved earlier and were lost in ferns.

Moss and hornwort SLAC homologues can be activated by OST1

Given the previously published finding of an active SLAC1-OST1 pair from the moss *P. patens* (PpSLAC1-PpOST1.2; Lind *et al.*, 2015), we further examined the evolution of SLAC activity using the distantly related moss *Sphagnum fallax* and the hornwort *Anthoceros agrestis* to determine whether other bryophytes share kinase-activated SLAC homologues. There has long been doubt over the phylogenetic relationship between hornworts and other land plants, but numerous recent studies have supported a monophyletic relationship between bryophytes (e.g. Leebens-Mack *et al.*, 2019; Harris *et al.*, 2020, 2022; Su *et al.*, 2021). We searched available genome sequences and identified the presence of a single SLAC/SLAH family member in *A. agrestis*, and two orthologs of *PpSLAC1* and three members of the OST1 subclade of SnRK2s in *S. fallax* (Table S4; Figs S4, S5), and were able to isolate all of these genes from gametophyte tissues. We found that both *S. fallax* and *A. agrestis* each possess a SLAC homologue that can be activated by ABA-signalling kinases in the *Xenopus* system (Fig. 4), similar to *P. patens* (Lind *et al.*, 2015). This indicates that among bryophytes, moss and hornwort species possess kinase-activated SLAC homologues, consistent with either an early origin for this mechanism, or independent gain/s in bryophytes.

Activation in gymnosperm and 'basal angiosperm' lineages requires auxiliary factors

Seed plants (gymnosperms and angiosperms) are the only plants to possess separate orthologs of Arabidopsis SLAC1, SLAH2/3 and SLAH1/4 that are strongly supported by phylogenetic analyses (Fig. S4; Dreyer *et al.*, 2012; Sussmilch *et al.*, 2019a). So far, the only seed plant SLAC1 channels to be examined have been from the core angiosperm lineages – dicots and monocots; SLAC1 activation has not previously been studied in gymnosperms and 'basal angiosperm' lineages. To further examine the evolution of SLAC1 activity in seed plants, we made use of the model gymnosperm *Picea abies*, which shows stomatal sensitivity to ABA, similar to angiosperms (Mayr *et al.*, 2012). The *P. abies* genome encodes four SLAC/SLAH proteins – PaSLAC1a, PaSLAC1b (co-orthologs of AtSLAC1), PaSLAH1 (an ortholog of AtSLAH1 and AtSLAH4) and PaSLAH2 (an ortholog of AtSLAH2 and AtSLAH3; Fig. S4; Table S4). Surprisingly, the *P. abies* SLAC1 co-orthologs failed to show S-type anion channel currents when co-expressed with OST1 in *Xenopus* oocytes (Fig. 5a).

We examined the protein sequences for amino acid differences that could account for the difference in activity between angiosperm and *P. abies* SLAC1 co-orthologs. We found that serine and threonine residues that are required (although not sufficient) for activation of SLAC1 proteins by OST1 and CPKs (Geiger *et al.*, 2009; Brandt *et al.*, 2015; Deng *et al.*, 2021) are missing in PaSLAC1a in the region corresponding to AtSLAC1 S120, and in PaSLAC1b in the region of AtSLAC1 S59 (Fig. 5b). Further examination showed that SLAC1 ortholog sequences of other species from the gymnosperm Pinaceae family similarly lack S120 (Fig. 5b). However, in species from other gymnosperm families, including *Ginkgo biloba*, a serine is present at these important positions. We tested the activity of this *G. biloba* SLAC1 (GbSLAC1a) and found that it could be activated by OST1 and CPKs (Fig. 5a,c). In contrast to the *P. abies* SLAC1 co-orthologs, WT PaSLAH2 (an ortholog of AtSLAH2 and

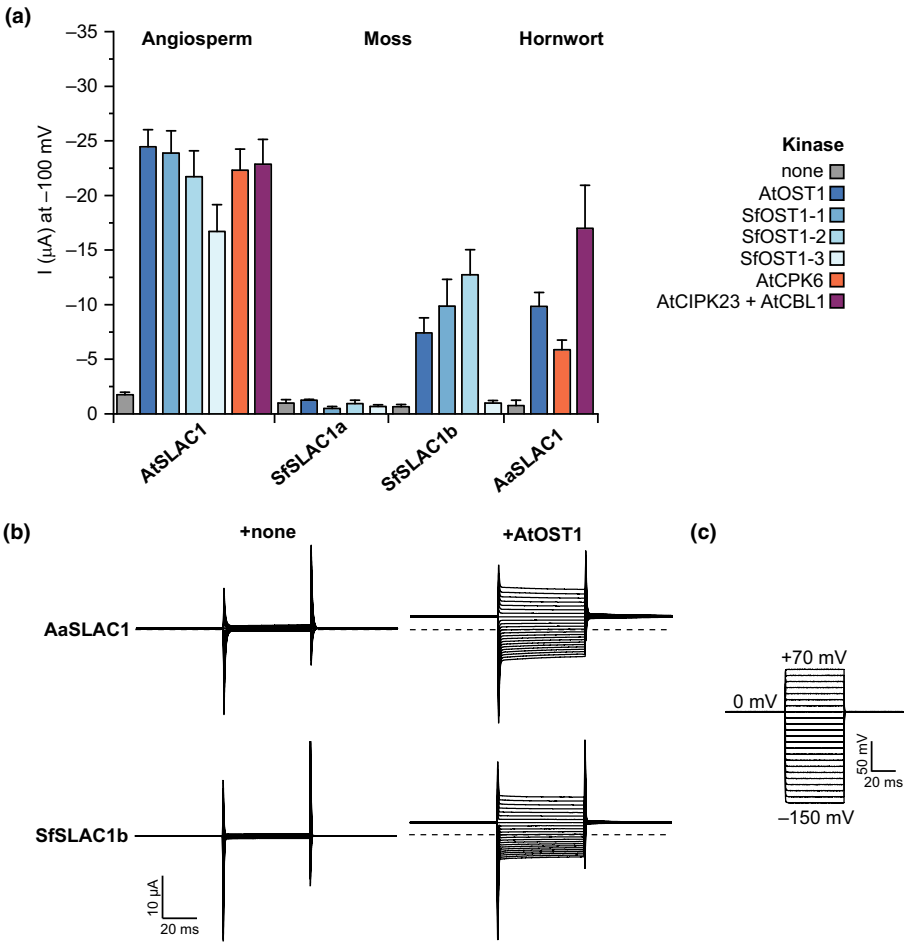

Fig. 4 Moss and hornwort SLOW ANION CHANNEL (SLAC) homologues can be activated by abscisic acid (ABA)-signalling kinases. (a) Mean whole-oocyte current measurements at −100 mV in nitrate-based solution with SLAC homologues from the angiosperm *Arabidopsis thaliana*, the moss *Sphagnum fallax* and the hornwort *Anthoceros agrestis*, co-expressed with or without the indicated kinases in *Xenopus* oocytes (mean ± SEM, $n \geq 4$). (b) Representative whole-oocyte currents of SLAC homologues from *A. agrestis* and *S. fallax* expressed either alone or with AtOST1, recorded in nitrate-based solution. (c) Voltage pulse protocol used for current measurements.

AtSLAH3) yielded substantial currents in nitrate-based media in the presence of a CPK (Fig. 5a), similar to AtSLAH3 (Geiger *et al.*, 2011). These results indicate that mechanisms for kinase activation of SLAC1 channels are present in some gymnosperms but have been lost from the Pinaceae family, where ABA-signalling kinases activate SLAH2/3 orthologs only.

To further examine the evolution of SLAC1 activity in seed plants, we made use of the single living representative of the Amborellales – the sister lineage to all other extant flowering plants – *Amborella trichopoda* (Albert *et al.*, 2013). The *A. trichopoda* genome encodes a single SLAC1 ortholog (AmtrSLAC1), two orthologs of SLAH2/3 and a single ortholog of AtOST1/AtSnRK2.2/AtSnRK2.3 (AmtrOST1; Figs S4, S5; Table S4). We examined the activity of AmtrSLAC1 when co-expressed with AtOST1, AmtrOST1 or AtCPK6, and found that it was insensitive to activation from any of these ABA-signalling kinases (Fig. 5a).

Discussion

We have found that guard cells of the fern models *C. richardii* and *P. vulgare* are equipped with ABA biosynthesis and signalling pathways (Figs 2, S2). Previous studies have suggested that the expression of ABA signalling and ion channel homologues in stomata-bearing tissues indicates a potential conserved role in ABA-mediated stomatal closure (e.g. Cai *et al.*, 2017; Plackett *et al.*, 2021). However, we have found that ABA-signalling kinases are unable to activate guard cell SLAC homologues from the ferns *P. vulgare* and *C. richardii* (Fig. 3). These results are consistent with our own results showing *P. vulgare* stomata to be insensitive to ABA application (Fig. 3), and previous findings of a lack of stomatal closure response to endogenous ABA levels in ferns (Brodribb & McAdam, 2011; McAdam & Brodribb, 2012; Cândido-Sobrinho *et al.*, 2022).

Furthermore, these results account for the lack of stomatal phenotype for ABA-insensitive fern mutants (McAdam *et al.*, 2016a). These fern mutants show sharp contrast to the wilty phenotypes characteristic of ABA biosynthesis and signalling mutants in angiosperm species with rapid stomatal closure in response to increases in endogenous ABA levels (Merlot *et al.*, 2002; Mustilli *et al.*, 2002; Merilo *et al.*, 2013; McAdam *et al.*, 2015). Further emphasising differences between the stomatal responses of seed plants and other vascular plant groups, there has been found to be a lack of ABA-mediated K^+ efflux from the guard cells of fern and lycophyte species (Gong *et al.*, 2021). Together, these results suggest that SLAC-like channels are not required for drought-induced stomatal closure in nonflowering plants, but instead may be involved in nutrient movement, similar to SLAH channels in

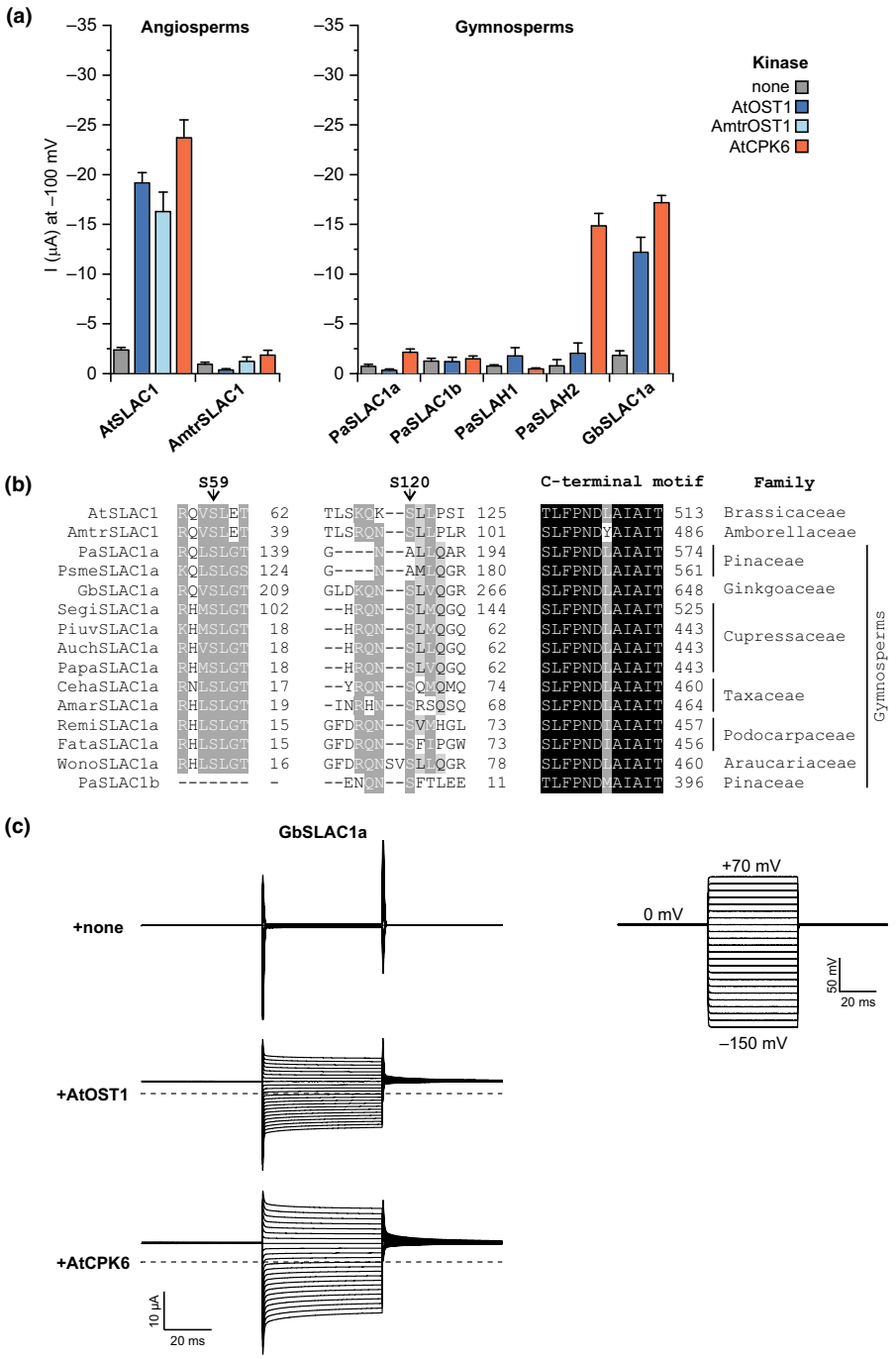

Fig. 5 Seed plants differ in terms of SLOW ANION CHANNEL 1 (SLAC1) activity, but SLAC1 HOMOLOGUE 2/3 (SLAH2/3) channel activity may be sufficient for sensitivity to abscisic acid (ABA)-signalling kinases. (a) Mean whole-oocyte current measurements at −100 mV in nitrate-based solution with SLAC/SLAH channels from the angiosperms *Arabidopsis thaliana* and *Amborella trichopoda*, and the gymnosperms *Picea abies* and the *Ginkgo biloba*, co-expressed with or without kinases in *Xenopus* oocytes (mean ± SEM, $n \geq 4$). (b) Alignment of protein regions surrounding AtSLAC1 residues S59, S120 and the C-terminal motif important for activation by ABA-signalling kinases, showing S59 is not conserved in PaSLAC1b, S120 is conserved in seed plants except members of the gymnosperm Pinaceae family, and the C-terminal motif is not conserved in AmtrSLAC1. Shading levels indicate the degree of conservation (black = 100%, dark grey ≥ 80% and light grey ≥ 60%). Numbers indicate the position in each protein sequence. Family is indicated for each species. Full sequence details are given in Supporting Information Table S4. (c) Example whole-oocyte currents of GbSLAC1a, either expressed alone (upper left), with AtOST1 (middle left) or with AtCPK6 (bottom left) recorded in nitrate-based solution using the indicated voltage pulse protocol (right).

angiosperms (Maierhofer *et al.*, 2014b; Cubero-Font *et al.*, 2016; Qiu *et al.*, 2016).

Ferns synthesise ABA in response to dehydration stress, similar to all other land plant groups (e.g. Hartung *et al.*, 1987; Hellwege *et al.*, 1994; Qin & Zeevaart, 1999; McAdam & Brodribb, 2012; Xiao *et al.*, 2018; Xu *et al.*, 2018), consistent with an ancient role for ABA in desiccation tolerance (e.g. Khandelwal *et al.*, 2010; Tougane *et al.*, 2010; Komatsu *et al.*, 2013; Jahan *et al.*, 2019; Shinozawa *et al.*, 2019). In response to slow dehydration, ABA triggers the upregulation of proteins that function in osmoregulation to protect fragile cellular components during desiccation, including metabolic enzymes, sugar transporters and aquaporins, in diverse plant species including bryophytes, lycophytes, ferns and angiosperms (e.g. Reynolds & Bewley, 1993; Hellwege *et al.*, 1994; Cuming *et al.*, 2007; Wang *et al.*, 2010; VanBuren *et al.*, 2017; Jahan *et al.*, 2019; Shinozawa *et al.*, 2019). In line with this function, ABA does induce changes in gene expression in ferns including in guard cells (Plackett *et al.*, 2021). Thus, although our results indicate that ABA signalling is not involved in rapid stomatal closure via ion channel activation as occurs in seed plants, it is likely that ABA signalling is involved in slow dehydration tolerance mechanisms in ferns, similar to other land plant lineages.

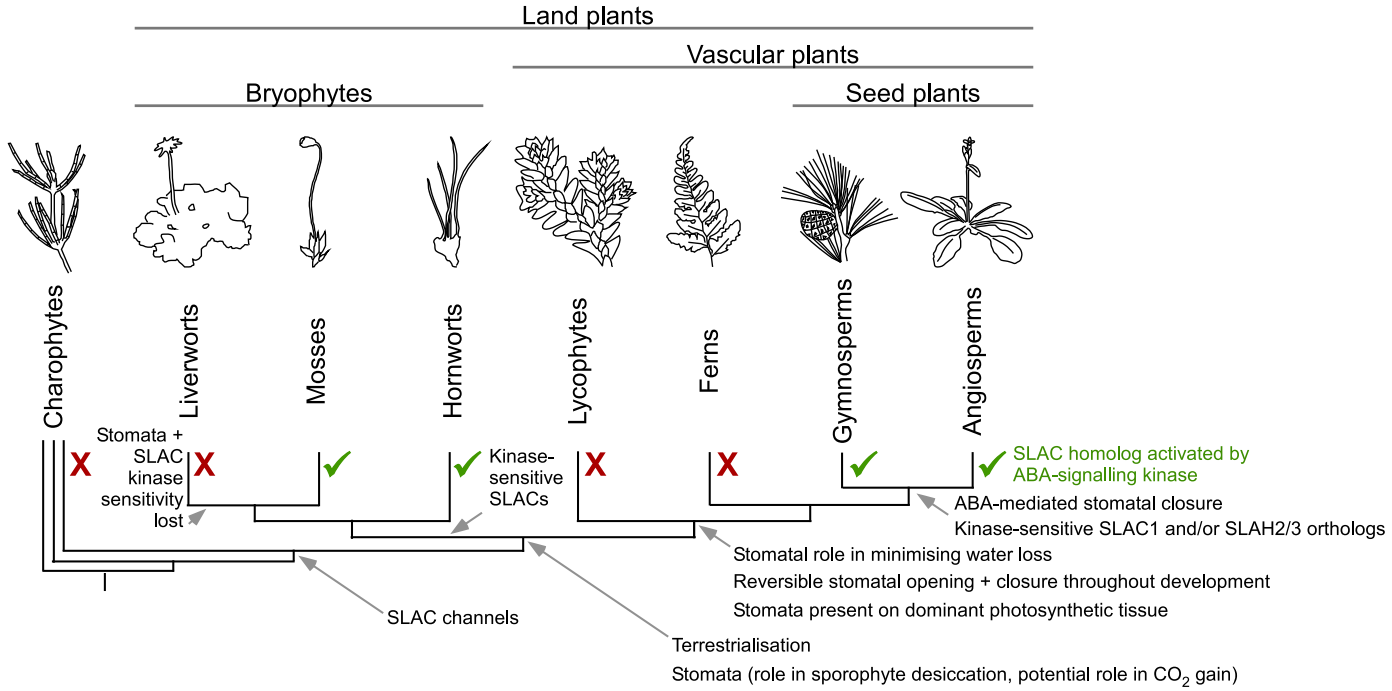

Fig. 6 Model for the timing of key events during stomatal evolution. The hypothesised timing of key traits is indicated with grey arrows on the current model of land plant phylogeny (Leebens-Mack *et al*., 2019; Harris *et al*., 2020). The presence or absence of evidence for SLOW ANION CHANNEL (SLAC) activation by abscisic acid (ABA)-signalling kinases for each major plant group is indicated with a tick or cross, respectively, based on the combined results of this study and previous findings (Geiger *et al*., 2009; Lind *et al*., 2015; McAdam *et al*., 2016a). Branch lengths are not to scale.

We find kinase-sensitive SLAC homologues in the moss *S. fallax* and the hornwort *A. agrestis* (Figs 3–5), where they might serve plant anion transport functions. It is not physically possible for SLAC homologues to play any role in stomatal closure in *S. fallax* and *A. agrestis*, as stomata 'open' by irreversible guard cell collapse in line with a predominant role for moss and hornwort stomata in promoting desiccation for spore drying and dispersal in these species (Duckett *et al*., 2009; Merced, 2015; Renzaglia *et al*., 2017). In line with an ancient role for ABA in vegetative desiccation tolerance (see McAdam & Sussmilch, 2021), application of exogenous ABA improves desiccation survival in *S. fallax* (Nibau *et al*., 2022). Although future mutant studies will be required to determine the precise roles of SLAC homologues in bryophytes, it is possible that a role for SLAC homologues in nutrient movement – similar to SLAH channels in angiosperms (Maierhofer *et al*., 2014b; Cubero-Font *et al*., 2016; Qiu *et al*., 2016) – may have been co-opted for ABA-dependent ion movement associated with desiccation tolerance in gametophyte tissues in mosses and hornworts.

We have found sensitivity to ABA-signalling kinases to be lacking for SLAC homologues from the alga *K. nitens*, liverwort *M. polymorpha* and lycophyte *S. moellendorffii*, in addition to the ferns *C. richardii* and *P. vulgare* and SLAC1 orthologs of seed plants *P. abies* and *A. trichopoda* (Figs 3–5; Lind *et al*., 2015; McAdam *et al*., 2016a). The lack of activation of the seed plant SLAC1 orthologs was particularly unexpected, given the shared ABA-mediated stomatal closure response in these angiosperm and gymnosperm species (Mayr *et al*., 2012; McAdam & Brodribb, 2015). However, it is possible that activation of SLAH2/3 orthologs by ABA-signalling kinases is sufficient for this response in these species.

Overall, we find evidence of a complex evolutionary history for SLAC sensitivity to ABA-signalling kinases with either (1) multiple gains or (2) an early gain for a nonguard cell-specific functional origin and subsequent losses (Fig. 6). Given the lack of this trait in lycophytes and ferns, we propose that bryophytes and seed plants may have separately co-opted SLAC/SLAH channels for different roles downstream of the ABA-signalling pathway, with seed plants using ABA to trigger rapid stomatal closure and some bryophytes using ABA for osmoregulation more generally in other cell types, including vegetative tissues that lack stomata. Our results explain differences in ABA sensitivity between some seed plants and ferns, while giving new insight into the transcriptional features of fern guard cells. These findings highlight the importance of studies directly testing if the roles of genes and the signal-dependent activation of encoded proteins are conserved in different plant species, in order to understand the evolution of plant signalling processes.

Acknowledgements

We thank Assoc. Prof. Scott McAdam and Prof. Tim Brodribb for assisting with clones of *C. richardii* and *P. abies* SLAC homologues and fruitful discussion, Dr Péter Szövényi and Prof. Jody Banks for early access to sequence data and provision of plant material, Dr Andrew Plackett for early sharing of sequence

information, and Prof. Stefan Rensing and Marco Göttig for provision of plant material. The support of the German Science Foundation (DFG) is acknowledged by MRGR, JS and RH for the project 'Evolution of molecular mechanisms that control stomatal closure' (RO2381/8-1, SCHU2352/7-1 and HE1640/40-1), by DB and RH for the DFG priority programme 2237: 'MAdLand – Molecular Adaptation to Land: plant evolution to change', by DG and RH for the CRC/TR166 'ReceptorLight' project B8 and by MM for the project TRR 356. PA and RH acknowledge funding by the Bavarian State Ministry of the Environment and Consumer Protection. KM acknowledges support from King Saud University's (KSU) Distinguished Scientist Fellowship Research Program (DSFP). FCS acknowledges support from the German Academic Exchange Service (DAAD) and an Australian Research Council Discovery Early Career Researcher Award (DE200101133) funded by the Australian Government.

Competing interests

None declared.

Author contributions

FCS, TM, JH, LJV, CL, MM, HMM, MSB and JS performed the research and analysed the data. FCS, PA, KFXM, DB, MRGR, DG, JS and RH designed the study. FCS, MRGR, DG, JS and RH wrote the manuscript.

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Data availability

Arabidopsis and fern sequences are accessible under the GenBank project accessions PRJNA731641 and PRJEB45027. Barley sequences are accessible at EMBL-EBI ArrayExpress under E-MTAB-10534. Orthogroup sequence details for genes of interest are listed in Table S6. All other gene accessions are listed in Table S4.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Most common annotated domains of differentially expressed orthogroups.

Fig. S2 Relative expression of orthogroups containing abscisic acid biosynthesis pathway components.

Fig. S3 Additional data for fern SLAC homologue activity and expression.

Fig. S4 Phylogeny of the SLAC/SLAH family in streptophytes.

Fig. S5 Phylogeny of the SnRK2 family in streptophytes.

Fig. S6 Bimolecular fluorescence complementation experiments showing interaction between fern SLAC homologues and kinases tested in oocytes.

Table S1 Overview of transcriptomes and differential gene expression analysis.

Table S2 Genomic data used for gene expression analysis and evolutionary reconstruction.

Table S3 Complete list of differentially expressed orthogroups from Fig. 1.

Table S4 Gene sequence details.

Table S5 Primer details.

Table S6 Orthogroup details for genes of interest for Fig. 2.

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